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Purification and Cloning of Interferon-Stimulated Gene Factor 2 (ISGF2): ISGF2 (IRF-1) Can Bind to the Promoters of Both Beta Interferon- and Interferon-Stimulated Genes but Is Not a Primary Transcriptional Activator of Either

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Inteferon-stimulated gene factor 2 (ISGF2) was purified from HeLa cells treated with alpha interferon. The factor, a single polypeptide of 56 kilodaltons (kDa), bound both to the central 9 base pairs of the 15-base-pair interferon-stimulated response element (ISRE) that is required for transcriptional activation of interferon-stimulated genes and to the PRD-I regulatory element of the beta interferon gene. ISGF2 was a phosphoprotein, and dephosphorylation in vitro reduced its DNA-binding activity. However, conditions that changed the amount of ISGF2 did not change the phosphorylated isoforms in vivo. ISGF2 in unstimulated cells existed in trace amounts and was induced by both alpha interferon and gamma interferon as well as by virus infection. Plasmid-bearing *Escherichia coli* clones encoding ISGF2 were selected with antibody against purified ISGF2. Sequence analysis revealed that the ISGF2 protein was the same as that encoded by the cDNA clone IRF-1, which has been claimed to activate transcription of interferon genes. We show that transcription of the ISGF2 gene was induced by alpha interferon, gamma interferon, and double-stranded RNA. However, ISGF2 was neither necessary nor sufficient for induced transcription of the beta interferon gene, while the factor NFκB was clearly involved.

Interferon-stimulated genes (ISGs) undergo a rapid transcriptional activation of greater than 100-fold in fibroblasts or HeLa cells treated with type I interferon (alpha interferon [IFN-α] or beta interferon [IFN-β]) (4, 13, 29, 47, 48). The induction peaks at about 1 h after treatment and declines to essentially baseline levels after as little as 6 h. If protein synthesis is prevented, the decline is considerably more gradual (13, 30). Most ISGs do not respond to gamma interferon (IFN-γ), but a few exhibit an immediate transcriptional response to either IFN-α or IFN-γ treatment (9, 50). The larger biological consequences of interferon treatment, slowing or cessation of cell growth and attainment of the antiviral state, occur within 24 h after treatment (for reviews, see references 42 and 53). In view of this signal-response program, two general kinds of induced proteins need to be examined: those connected with the original transcriptional response, and those that ultimately lead to the antiviral state or growth inhibition.

To begin to study the first group of proteins, we cloned the promoters of two ISGs (33, 47) and identified the interferon-stimulated response element (ISRE) (34, 47), a 15-base-pair (bp) highly conserved enhancer element within a larger, less-conserved region of homology found in some ISGs (14). The ISRE was shown to be necessary and sufficient for ISG transcriptional induction by IFN-α (26, 34, 47, 49). The ISRE region from one IFN-α-stimulated gene also contained sequences required for IFN-γ-stimulated transcription (50); although we have recently found that the IFN-γ-responsive element is not limited to the IFN-α ISRE (D. J. Lew, T. Decker, and J. E. Darnell, Jr., unpublished data).

Three distinct protein-DNA complexes can form in vitro with nuclear extracts from IFN-α-treated cells plus the ISRE of ISG54 or ISG15 (27, 34, 35, 49). A number of additional ISGs are also known to contain a very similar ISRE that forms the same or similar protein-DNA complexes (6–8, 23, 45, 51). Constitutive and interferon-inducible DNase-hypersensitive sites near the ISRE in both ISG15 and ISG54 suggest that the proteins that form these complexes are significant in vivo (44).

We have called the three factors that form these complexes interferon-stimulated gene factors (ISGFs). (i) ISGF3, like IFN-α-stimulated ISG transcription, is rapidly induced upon treatment of cells with type I interferon, even in the absence of protein synthesis (30, 34). Both binding of ISGF3 and transcriptional induction of the ISGs require the entire ISRE (26). For these reasons, and based on many additional experiments (27, 36, 44, 49; X.-Y. Fu and J. E. Darnell, Jr., unpublished data; D. E. Levy, unpublished data), we have concluded that ISGF3 is the activator of interferon-dependent ISG transcription. (ii) ISGF1 is a constitutive factor. (iii) ISGF2 is induced in response to IFN-α, but induction requires protein synthesis (34). Both ISGF1 and -2 require only the central 9 base pairs of the ISRE for binding (26, 36, 49). Thus, neither protein could be the primary activator of ISG transcription.

The promoter region of the IFN-β gene contains several protein-binding sites (11, 16, 25), one of which, PRD-1, is homologous to the central core of the ISRE (20, 55). It has been claimed that a factor, IRF-1, that recognizes this core element is sufficient to activate transcription of the IFN-β gene in vivo (17, 21). It has also been implied that this putatively positive-acting factor may mediate interferon-stimulated transcriptional increases (16, 21, 40). The strongest support for these conclusions was cotransfection experiments with artificial target constructs containing polymerized oligonucleotide hexamers that behaved like a

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virus-inducible element (15) and happened to reconstitute an ISRE (46), and another plasmid to express IRF-1.

To address questions about the functional mechanism and the role of induced proteins that bind to the core of the ISRE, and thus probably to the PRD-1 element, we have purified ISGF2 from HeLa cells treated with IFN-α. This single purified protein did bind not only to the core of the ISRE, but also directly to the PRD-1 element of the IFN-β promoter. The protein preexisted at a low basal level and was induced by virus infection or treatment with double-stranded RNA (dsRNA) or IFN-γ, as well as with IFN-α. ISGF2 was found to be a phosphoprotein, but the level of DNA-binding activity reflected the amount of protein, while the distribution of phosphorylated isoforms was always essentially the same. ISGF2 cDNA clones selected from a human cDNA expression library with a specific antibody were found to be the same as human IRF-1 (37). The ISGF2 gene is transcriptionally induced by IFN-α, IFN-γ, and dsRNA. However, large amounts of ISGF2 did not necessarily induce chromosomal IFN-β gene transcription. In fact, IFN-β induction could occur even when ISGF2 was undetectable. While these data cannot rule out an ancillary role for ISGF2 in transcriptional activation of either the IFN-β gene or the ISGs, this protein cannot be the major activator for these genes. These data highlight the importance of determining what does happen to endogenous genes as well as what can happen to transfected artificial constructs before assigning a function to a transcription factor.

MATERIALS AND METHODS

Cell growth, extract preparation, and ISGF assay. For protein purification, HeLa-S3 cells (ATCC CC1 2.2) grown as suspension cultures in Joklik modified minimal Eagle medium (GIBCO) plus 5% calf serum (Hyclone) received 500 U of human recombinant IFN-α (Hoffmann-La Roche) per ml for 2 h prior to harvest. Otherwise, the cells were grown as monolayer cultures in Dulbecco modified Eagle medium plus 5% calf serum. Other treatments with recombinant IFN-α, recombinant human IFN-γ (Amgen), dsRNA [poly(I):poly(C); Pharmacia], or cycloheximide (CHX) were as specified in the figure legends. Newcastle disease virus (NDV), a gift from P. Seghal, Rockefeller University, was used where indicated in the figure legends at a multiplicity of infection of 10.

Nuclear extracts were prepared essentially as described before (10) except that nuclei were pelleted at 3,000 × *g* after cell lysis and then suspended with two-thirds volume of EB (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.9], 1 mM dithiothreitol [DTT], 0.4 mM phenylmethylsulfonyl fluoride [PMSF], 20% glycerol) and adjusted to 0.4 M NaCl with EB plus 2.4 M NaCl. Extracts were dialyzed against ECB(0.2) (20 mM HEPES [pH 7.9], 1 mM DTT, 0.4 mM PMSF, 10% glycerol, 0.2M KCl; ECB(*n*) is ECB plus *n* M KCl), clarified, frozen in liquid N_2, and stored at less than −80°C. Protein was measured with a Bio-Rad Laboratories kit, and ISGF activity was determined by the electrophoretic mobility shift assay (EMSA) (12) in a previously described reaction mixture (34) with an ISG15 promoter fragment (26, 49).

Radiolabeled oligonucleotides and unlabeled oligonucleotide competitors were used for certain EMSAs as described in the figure legends. These reactions were electrophoresed on 6% polyacrylamide gels. The oligonucleotides were the ISG15 promoter sequence from −117 to −89, which includes the ISRE (47), flanked by *Bam*HI sites; the IFN-β promoter sequence from −77 to −54, which includes the regulatory elements PRD-I and PRD-II (20), flanked by *Sau*3AI sites; the immunoglobulin κ gene enhancer sequence (38) from +3929 to +3953, which includes the binding site for the factor NFκB (52), flanked by *Bam*HI sites; or the adenovirus type 5 E2 gene sequence from −76 to −30, which contains the repeated binding sites for the E2F protein (56), flanked by *Sau*3AI sites.

Purification of ISGF2. Chromatographic separations were carried out at 4°C. Active fractions were detected by EMSA, and except as specified, fractions or pools were frozen and stored as above. Heparin-agarose (Sigma, type I) equilibrated in ECB(0.2) was loaded with extract from 26 liters of HeLa cell culture (up to 10 mg of extract protein per ml of bed volume), washed with 3 column volumes of starting buffer and then with 3 column volumes of ECB(0.32), and finally eluted with a 6-column-volume linear gradient from 0.32 to 0.6 M KCl in ECB. Fractions with ISGF2 activity (0.42 to 0.47 M KCl) were pooled, precipitated with 50% $(NH_4)_2SO_4$, dissolved in OCB(0) [10 mM Tris (pH 8.0), 1.5 mM $MgCl_2$, 1 mM DTT, 0.1% Nonidet P-40, 10% glycerol; OCB(*n*) is OCB plus *n* M KCl], frozen, and stored as above. The heparin-agarose pool was adjusted with OCB(0) to a conductance of 360 μS/cm (equivalent to 0.2 M KCl) and loaded onto 2 ml of nonspecific oligonucleotide-Sepharose that had been prepared with a simian virus 40 core C sequence (TTTTGGGACTTTCCACACCCTAAC annealed to TTTTGTTAGGGTGTGGAAAGTCCC) and cyanogen bromide-activated Sepharose (Pharmacia) as described before (24). The flowthrough was immediately adjusted to 8 μg of poly(dIdC:dIdC) per ml and 2 μg of pGem1 plasmid per ml and loaded onto 2 ml of a specific oligonucleotide-Sepharose that had been made as above with a ligated ISG15 ISRE (GGATCCAAAGGGAAACCGAAACTG annealed to GG ATCCCAGTTTCGGTTTCCCTTT). After extensive washing with OCB(0.2), ISGF2 was eluted with 8 ml of OCB(0.8) and 4 ml of OCB(1.0). The column was recycled by extensive washing with OCB(2.0) and reequilibrating with OCB(0.2). Active fractions were pooled and diluted to 0.2 M KCl with OCB(0). Competitor DNA, as above, was added to one-fourth the previous concentrations, and the sample was rechromatographed in the same way on the same column. The active fractions were pooled, diluted to 0.1 M KCl with OCB(0), and loaded on a 1 ml Mono-Q fast protein liquid chromatography column (Pharmacia) equilibrated with OCB(0.1). After being washed with additional OCB(0.1), the column was brought to OCB(0.45) with a 20-ml linear gradient. Active fractions (0.25 to 0.35 M KCl) were pooled, and pools from four Mono-Q columns were concentrated batchwise with Q-Sepharose (Pharmacia). Fractions or pools were frozen and stored as described above after each specific oligonucleotide step and after separation on Mono-Q or concentration on Q-Sepharose.

Characterization of ISGF2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described before (28) to analyze the purification and for other experiments as indicated. Samples were adjusted to be in the same volume of the same buffer, concentrated with Centricon 30 ultrafilters (Amicon), and then adjusted to 1× SDS-PAGE loading buffer (28) before recovery of the retentate to minimize loss on the filter membrane. Renaturation of ISGF2 after SDS-PAGE was done as described before (5), except with the addition of 0.1 mg of bovine serum albumin (BSA) per ml as carrier for elution from the polyacrylamide gel.

For DNase footprinting, 5% glycerol was substituted for

TABLE 1. Purification of ISGF2

Fraction[a]	Volume (ml)	Protein[b] (μg)	Activity[c] (U)	Sp act (U/μg)	Yield (%)		Purification (fold)	
					Per step	Cumulative	Per step	Cumulative
Extract	95.5	95,606	18,256	0.19	100	100	1	1
Heparin-agarose	4.4	5,158	18,844	3.65	103	103	19	19
1st oligonucleotide	8.7	54	11,554	214	61	63	59	1,126
2nd oligonucleotide	8.4	17	7,739	455	67	42	2	2,396
Mono-Q	8.4	1	4,788	4,788	62	26	11	25,200

[a] The flowthrough fraction from a nonspecific oligonucleotide affinity column that was loaded directly onto the first-pass specific oligonucleotide affinity column is not shown.

[b] Protein quantitation was based on the electrophoretic analysis of the fractions shown in Fig. 1. The integrated densitometric signal from the known amount of protein in the marker lane was used to obtain an average value for silver stain signal per microgram of protein. The integrated signal from each sample lane was normalized to that value and to the proportion of each fraction used for the analysis (see legend to Fig. 1).

[c] One unit binds 1 fmol of specific probe under standard EMSA conditions (see Materials and Methods).

Ficoll in the standard EMSA binding reactions. Either 1.0 μg of BSA or various amounts of purified ISGF2, as indicated in the legend to Fig. 3, were mixed with 2 ng of labeled ISG15 or ISG54 promoter fragments and a 500-fold molar excess of nonspecific oligonucleotide or, where indicated, a 500-fold molar excess of a homologous ISRE oligonucleotide. The samples were incubated for 30 min at 22°C and then for 30 min at 4°C. Finally, 0.1 volume of DNase I, at 20 ng/μl in 10 mM $CaCl_2$, was added, and incubation was continued for an additional 4 min at 4°C. The reactions were stopped with excess EDTA and proteinase K digestion, DNA was recovered and electrophoresed on 8% acrylamide–8 M urea denaturing gels, and the gels were dried and autoradiographed.

Immunoblots. Purified ISGF2, as a single band after SDS-PAGE, was used to raise a rabbit polyclonal antiserum. Proteins were resolved by SDS-PAGE or in two dimensions by isoelectric focusing and SDS-PAGE as described before (41) and then transferred to Immobilon-P membrane (Millipore) by electroblotting at 4°C in 25 mM Tris base–192 mM glycine–15% (vol/vol) methanol with a Bio-Rad Laboratories Minitransblot device. Antigens were detected with preimmune or anti-ISGF2 antiserum, diluted as indicated in the figure legends, and an alkaline phosphatase-conjugated "AffiniPure" $F(ab')_2$ fragment of goat anti-rabbit immunoglobulin G (Jackson Immunoresearch Laboratories), diluted 1:2,500, as the second antibody. Membranes were blocked, incubated with antibodies, and washed in 10 mM Tris (pH 8.0)–150 mM NaCl–0.05% Tween 20–2.5% nonfat dry milk. Milk was omitted from the washes after incubation with the second antibody. The color reaction was done in 100 mM Tris (pH 9.5)–100 mM NaCl–5 mM $MgCl_2$, with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, *p*-toluidine salt (Sigma).

Cloning of ISGF2. An oligo(dT)-primed cDNA library of 2.5×10^6 recombinants was prepared in λZAP II (Stratagene). The template was polyadenylated RNA isolated from HeLa cells that had been treated with IFN-γ and IFN-α. An optimal-sequence oligonucleotide (31) was designed based on the amino acid sequence of ISGF2. Its reverse complement, 5'-GGGATCTGGTTGGAGTTGATCTGCATCTCCAG CCAGGGCCGCATCCGCAT-3', was used to screen 500,000 clones. Among 276 positive clones, 64 were further screened with anti-ISGF2 antiserum. Nine clones were plaque purified based on immunoreactivity. The inserts were recovered in the vector pBluescriptSK$^-$ according to the supplier's instructions. Eight of the clones appeared to be independent, and five were characterized in detail.

Determination of transcription rates. Run-on assays were performed with isolated nuclei to determine relative rates of transcription as described previously (29). Plasmids containing an ISGF2 cDNA and other plasmids previously described in detail (9, 43) were used as probes. These included exon fragments of ISG15 and ISG54 genomic clones, a full-length IFN-β cDNA clone (gift of E. Knight, Du Pont), a guanylate binding protein (GBP) full-length cDNA clone, actin or tubulin probes for normalization, and pGEM1 (Promega) as a negative control.

RESULTS

Purification of ISGF2 from cells treated with IFN-α. Table 1 shows the steps used in purification of ISGF2. Approximately 100 mg of protein was extracted from the nuclei of approximately 10^{10} HeLa cells. The purification was monitored by EMSA. The characteristic complex formed by ISGF2 with a probe from the promoter of the ISG15 gene (26, 49) served to identify the active fractions (data not shown). From the starting material, 1 μg of ISGF2 was obtained. The cumulative yield of activity was 26%, and the final purification was at least 25,000-fold. An independent quantitation by Coomassie blue staining of nuclear extract protein and the purified ISGF2 indicated a purification of 70,000-fold.

A portion from each fraction listed in the table was examined by SDS-PAGE (Fig. 1). Even in the heparin-agarose fraction, a protein(s) migrating at 56 kilodaltons (kDa) was enriched. The relative abundance of different species was greatly altered after the first pass through the specific oligonucleotide affinity column, and the second pass reduced the remaining major contaminants. After the final step, anion-exchange chromatography, a protein of 56 kDa was the only major component detected by silver stain. When the yield from several independent preparations was pooled, concentrated, and used for preparative SDS-PAGE, Coomassie blue stain revealed only a minor contaminant of approximately 43 kDa and a few very faint bands of greater than 97 kDa. The material migrating as a 56-kDa species composed at least 90% of the final preparation.

When the 56-kDa protein was eluted after SDS-PAGE (Fig. 2, slice 4) and renatured, DNA-binding activity identical to that of the input ISGF2 was recovered. In addition, DNase protection experiments (Fig. 3) performed on both strands of fragments that contained the essential regulatory and promoter elements of either ISG15 or ISG54 (33, 47) showed that the purified protein specifically protected only a small area of each strand, which encompassed the ISRE (compare lanes 4 and 5 to lane 6, or lanes 11 and 12 to lane 13 in panel A and panel B). No other sites in these fragments were bound by the purified preparation of ISGF2 (data not shown). Figure 2C shows schematically that protection with

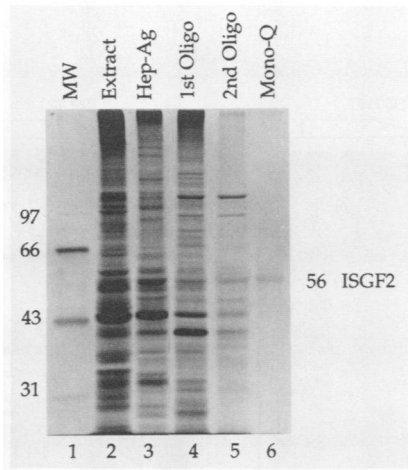

FIG. 1. ISGF2 was purified from the nuclei of HeLa cells that had been treated with IFN-α. Samples were prepared and electrophoresed until the bromophenol blue reached the bottom on an 8% separating gel, as described in Materials and Methods, and then protein was detected by silver staining with a Bio-Rad kit. Lanes: MW, 100 ng of the Bio-Rad low-molecular-weight marker set per protein, with the size (in kilodaltons) indicated alongside; extract, 0.0025% of crude nuclear extract prepared from approximately 10^{10} IFN-α-induced HeLa cells; Hep-Ag, 0.025% of the active fraction recovered after heparin-agarose chromatography and ammonium sulfate precipitation; 1st Oligo, 2.5% of the active pool from the first pass through the specific oligonucleotide affinity column; 2nd Oligo, 2.5% of the active pool from the second pass through the specific oligonucleotide column; Mono-Q, 2.5% of the active fractions pooled after chromatography on the Mono-Q anion exchanger. Purified ISGF2 is indicated with its apparent size at the right. The samples were taken from a single typical preparation.

respect to each ISRE was very similar, although the ISREs are in opposite orientations in these two genes relative to the direction of transcription. From these experiments we conclude that the single purified protein of 56-kDa apparent molecular weight was responsible for the DNA-binding activity of the previously identified factor ISGF2 (34, 35).

ISGF2 binds to both the ISRE and the IFN-β promoter. Suggestions have been made that the regulation of ISGs and type I interferon genes might employ the same protein factors (16, 21, 40). We showed earlier that while the binding to the ISRE of ISGF3 (the activator of ISG transcription) is not competed with by regulatory elements from the IFN-β promoter, the binding of ISGF2 in crude extracts was affected (36). The purified ISGF2 also bound directly to the PRD-I element of the IFN-β promoter when assayed by EMSA in the presence of nonspecific competitor (Fig. 4, lane 5), as of course it did to an ISRE oligonucleotide (lane 1). Since the specific activity and concentration of the IFN-β and ISRE probes, as well as the amount of ISGF2, were the same in these assays, it is clear that ISGF2 bound with higher affinity to the ISG15 ISRE than to the PRD-I element in the IFN-β oligonucleotide.

The IFN-β oligonucleotide also contained a PRD-II element that is an NFκB binding site (18, 22, 32, 54). However, as tested by competition with an unlabeled NFκB-specific oligonucleotide (Fig. 4, lane 6) or by use of a labeled NFκB probe (data not shown), it was apparent that ISGF2 bound to the IFN-β oligonucleotide due to the PRD-I element and not the NFκB site and that ISGF2 could not bind to an NFκB site. These data constitute the first proof that a single protein purified from a mammalian source binds to sequences in both ISGs and IFN-β genes.

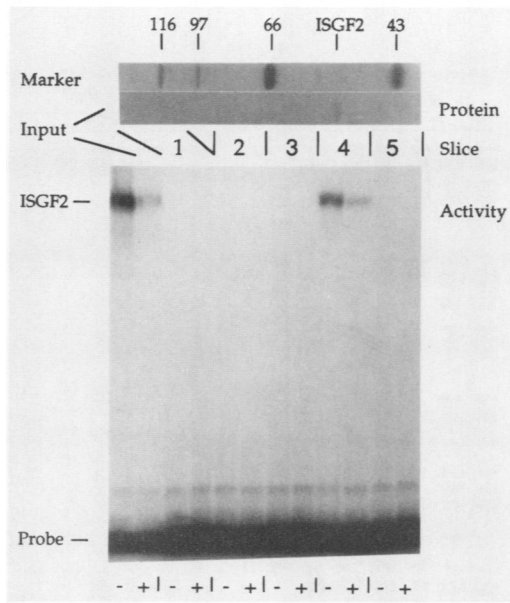

FIG. 2. The purified 56-kDa protein renatures after SDS-PAGE and has ISGF2 activity. Bio-Rad high-molecular-weight markers and two samples of ISGF2 from a Mono-Q fraction were prepared and electrophoresed, as described in Materials and Methods, until the 43-kDa marker was near the bottom of an 8% separating gel. (Top) The silver stain of the markers and one lane of sample, with the molecular sizes (kDa) and the location of ISGF2 indicated above, are shown horizontally. (Bottom) A sample of the Mono-Q fraction used for the SDS-PAGE separation and samples of the protein(s) eluted and renatured from slices of the unstained sample lane were tested for ISGF2 activity by EMSA with the ISG15-130 promoter fragment as the probe. In some lanes (−), 0.5 μg of pGEM1 was used as nonspecific competitor DNA. In other lanes (+), 0.5 μg of a recombinant plasmid containing the ISG15 promoter sequence was used in place of the pGEM1, which provided a 40-fold molar excess, relative to the probe, of specific competitor DNA. The location of the free probe and the characteristic complex with ISGF2 are indicated. The correspondence between slices from the unstained sample lane and the pairs of lanes from the EMSA are indicated relative to the stained sample and markers.

Antibody against ISGF2 does not recognize ISGF1 or ISGF3. An antibody was raised against purified, IFN-α-induced ISGF2. Specificity of the immune serum was confirmed by immunoblot analysis of each ISGF2 fraction from the purification scheme (data not shown). In addition, the ISGF2-DNA complex interacted with the antiserum, while complexes formed from ISGF1 or ISGF3 were not recognized (Fig. 5). (During purification, it was found that high concentrations of ISGF2, such as were present in this extract, gave rise to the specific complex seen in lane 1 between the complexes formed by ISGF2 and ISGF3 [unpublished data].) Preimmune serum had a negligible effect on the mobility of any of the complexes (lane 4), while immune serum caused dramatic retardation specifically of the ISGF2 complex (lane 5). When formation of protein complexes with labeled oligonucleotide was largely prevented by specific oligonucleotide competitor, the immune serum did not cause the appearance of any significant new complex (lanes 6 and 7). In the presence of preimmune serum, nothing was specifically removed by fixed *Staphylococcus aureus* cells (lane 8). With immune serum, the highly retarded material was immunoprecipitated, which proved that it was an immune complex (lane 9). The same incubations had no visible effect on ISGF1 in several separate samples, e.g., extracts

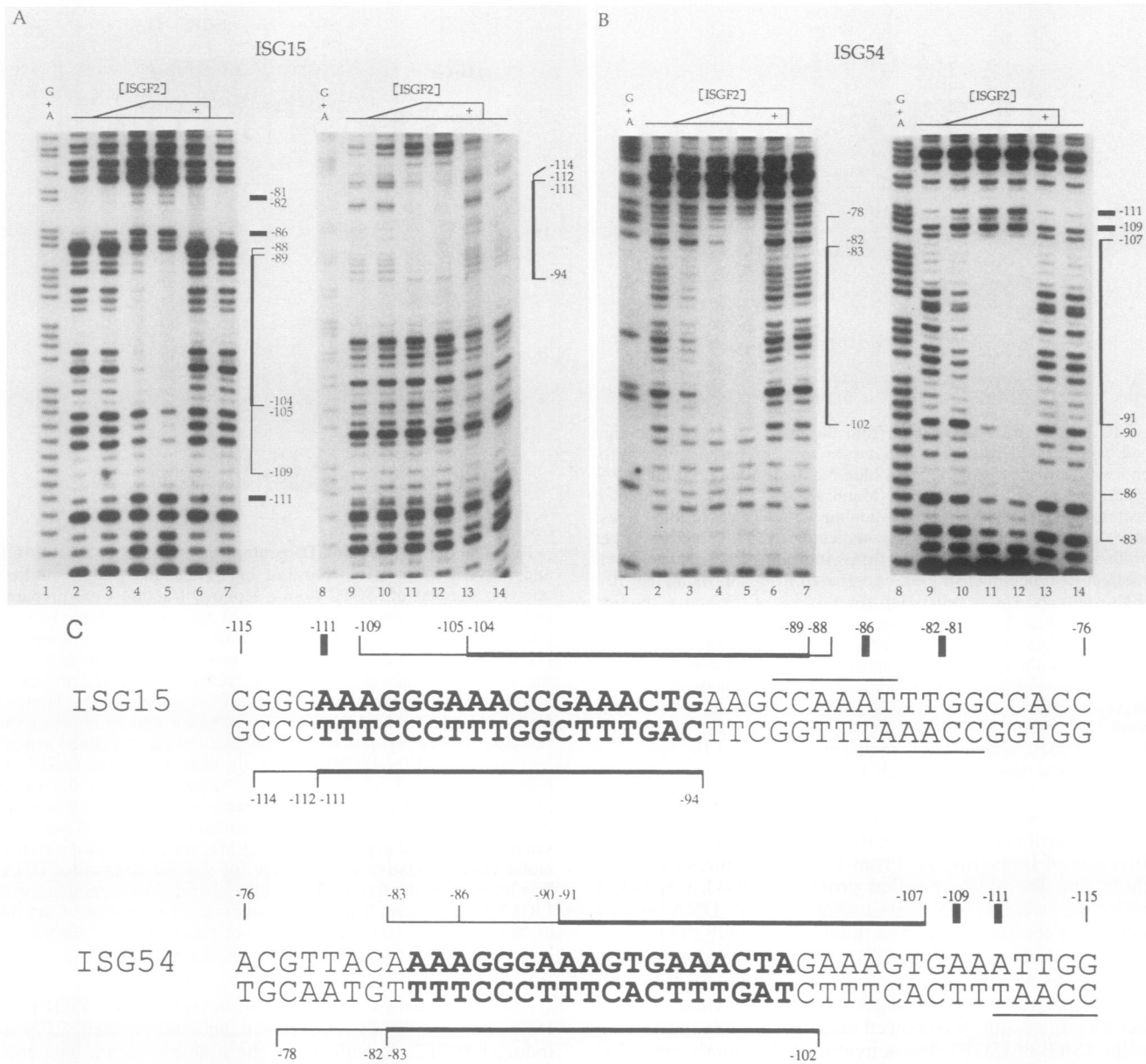

FIG. 3. Purified ISGF2 binds only to the ISRE region in ISG15 and ISG54 promoter fragments. (A) A 130-bp fragment including from −115 to −37 of the ISG15 promoter flanked by *Bam*HI linkers and the p*GEM*1 polylinker was 5′-end labeled at either the upstream end of one strand (left panel) or the downstream end of the other strand (right panel). Upstream and downstream are with respect to the direction of transcription. Samples without ISGF2 had BSA instead, and samples without specific oligonucleotide had a nonspecific oligonucleotide. Lanes 1 and 8, G-plus-A ladder was generated as described before (39) from the respective end-labeled fragments; lanes 2, 7, 9, and 14, no ISGF2; lanes 3 and 10, 0.8 ng of ISGF2; lanes 4 and 11, 1.6 ng of ISGF2; lanes 5 and 12, 3.0 ng of ISGF2; lanes 6 and 13, 3.0 ng of ISGF2 plus a 500-fold molar excess of specific competitor DNA. The positions of changes in the DNase cleavage pattern are indicated to the right of each panel. Thick bars indicate DNase hypersensitivity caused by ISGF2; thin and thick lines are drawn alongside sequences partially or completely protected, respectively, against DNase digestion. (B) A 176-bp fragment including the ISG54 promoter and first exon, from −122 to +54, was labeled and treated, and the results are shown as described for panel A. (C) The DNase footprint of ISGF2 around the ISRE in ISG15 or ISG54 is depicted schematically, as described for panel A, in relation to the nucleotide sequence of each strand. The ISRE is shown in boldface type, and possible or likely CCAAT boxes are marked by a line adjacent to the possible or likely CCAAT boxes.

from untreated or IFN-α-treated cells, that had less ISGF2 than the sample used for this experiment (data not shown; see figure legend).

ISGF2 is regulated by changes in abundance, not by changes in DNA-binding affinity. With the availability of ISGF2-specific antiserum, qualitative and quantitative analyses of ISGF2 were performed after various treatments of the purified protein or of cells that were then used to prepare nuclear extracts (Fig. 6). The first experiment (Fig. 6A) examined whether phosphorylation might contribute to ISGF2 DNA-binding activity. Two-dimensional separation by isoelectric focusing and SDS-PAGE resolved pure ISGF2 into three major isoforms (pIs, ca. 5.3 to 4.7) (left side). Incubation of the factor with potato acid phosphatase converted the ISGF2 to a predominant spot that comigrated with the most basic of the native isoforms (right side). This treatment reduced

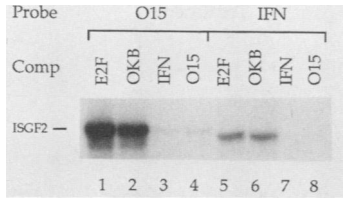

FIG. 4. Purified ISGF2 binds not only the ISRE core, but also to the PRD-I element of the IFN-β promoter. EMSA was performed with 0.3 ng of pure ISGF2 and radiolabeled oligonucleotides that included either the ISG15 ISRE (O15, lanes 1 to 4) or the PRD-I and PRD-II elements of the IFN-β promoter (IFN, lanes 5 to 8). The complex between ISGF2 and DNA, but not free DNA, is shown. Unlabeled oligonucleotides that contained either specific binding sites for the transcription factor E2F (lanes 1 and 5) or NFκB (lanes 2 and 6), or that were the same as the IFN probe (lanes 3 and 7) or the O15 probe (lanes 4 and 8) were included at a 200-fold molar excess. The DNA sequence included in each oligonucleotide is described in Materials and Methods.

DNA-binding activity perhaps three- to fivefold (Fig. 6B), indicating that the relative affinity but not the specificity for binding to the ISRE might be affected by phosphorylation. In the presence of phosphatase inhibitors, the reduction was prevented, clearly linking the dephosphorylation detected by isoelectric focusing to the decrease in ISGF2 DNA-binding activity upon treatment with phosphatase. Inclusion of 30 mM $Na_2P_2O_7$ in the incubations did not prevent the decrease in ISGF2 DNA-binding activity, but in combination with 50 mM NaF, 100 μM Na_3VO_4, or both the loss of activity was substantially or completely blocked (data not shown).

Whether qualitative or quantitative changes in ISGF2 occurred in cells treated in various ways was determined next. Qualitative differences might allow different roles for ISGF2 induced by different agents. Since ISGF2 bound to the virus-inducible PRD-I element of the IFN-β promoter, induction of ISGF2 by NDV was tested, as was the induction of ISGF2 by IFN-α or IFN-γ (9, 36). None of these treatments altered the steady-state distribution of ISGF2 isoforms that existed in untreated cells (Fig. 6C). Since extracts prepared in the presence of a battery of phosphatase inhibitors (50 mM NaF, 30 mM $Na_2P_2O_7$, 100 μM Na_3VO_4) still had the same distribution of ISGF2 isoforms (data not shown), this pattern almost certainly reflects the actual in vivo phosphorylation state of ISGF2. This conclusion was supported by multiple analyses of these and other extracts (data not shown). While the proportion of each isoform remained essentially the same, immunoblot analysis after SDS-PAGE alone showed that the overall abundance of ISGF2 differed among the samples (Fig. 6D). An EMSA (Fig. 6E) showed that the level of ISGF2 DNA-binding activity paralleled the amount of ISGF2 in each nuclear extract. Therefore, it is unlikely that differential phosphorylation is a mechanism for regulating the role of ISGF2; rather, ISGF2 function may be regulated by the abundance of the protein.

ISGF2 levels are transcriptionally regulated. ISGF2 clones were isolated from a human cDNA expression library by using an oligonucleotide probe based on the ISGF2 amino acid sequence and then an antibody against ISGF2 (see Materials and Methods). Nucleotide sequence analysis of several independent isolates (data not shown) revealed that ISGF2 is almost certainly identical to the recently reported human clone called IRF-1 (37). With an ISGF2 cDNA probe, it was found that the mRNA abundance could account for the relative protein levels in untreated cells, cells infected with NDV, and cells treated with either IFN-α or IFN-γ. Also, CHX caused increased accumulation or superinduction of the mRNA (unpublished data).

Nuclear run-on assays (Fig. 7) were performed to determine whether regulation of the rate of ISGF2 transcription was ultimately responsible for changes in the levels of ISGF2 DNA-binding activity or mRNA concentrations. A low constitutive level of ISGF2 transcription was observed; IFN-α or CHX elevated ISGF2 transcription approximately three- to fivefold, while IFN-γ induced ISGF2 transcription 10- to 20-fold. The specificity of the response to each treatment was verified by control probes. As expected, IFN-α induced ISG15 and ISG54 transcription far above the undetectable basal level (29, 48), while relative to the tubulin and actin controls, there was no response by these genes to IFN-γ (29). In contrast, the GBP gene exhibited its typical response to both IFN-α and IFN-γ induction (9). A role for ISGF2 in regulating any of the ISGs or the IFN-β gene was not established by this experiment, even though all of these genes contained binding sites for the protein; however, the ISGF2 gene was clearly regulated by interferons.

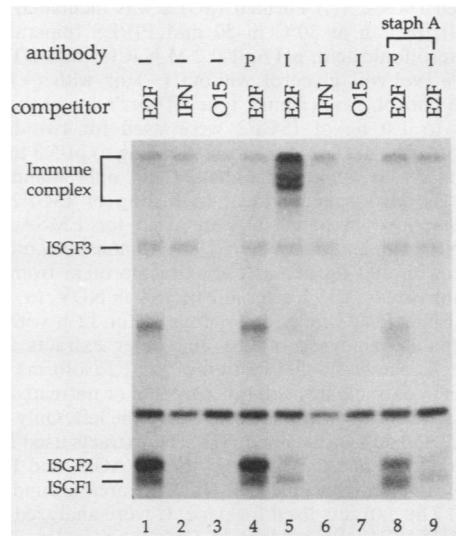

FIG. 5. Antibody raised against pure ISGF2 does not cross-react with ISGF1 or ISGF3. EMSA binding reactions were performed with a radiolabeled ISG15 ISRE oligonucleotide (O15) and 3 μg of nuclear extract from HeLa cells that had been treated with IFN-γ (1 ng/ml) for 12 h and IFN-α (500 U/ml) for 2 h. Unlabeled oligonucleotide that contained either a specific binding site for an unrelated transcription factor (E2F, lanes 1, 4, 5, 8, and 9) or the PRD-I and PRD-II elements of the IFN-β promoter (IFN, lanes 2 and 6), or was the same as the O15 probe (lanes 3 and 7) was included at a 100-fold molar excess. Inclusion of additional binding buffer (lanes 1 to 3), preimmune serum (P, lanes 4 and 8), immune serum (I, lanes 5 to 7 and 9), or fixed *S. aureus* cells (staph A, lanes 8 and 9) was as follows. One-fifth volume (2.5 μl) of additional binding buffer or serum diluted 20-fold in binding buffer was added and incubation was continued for 1 h at 4°C. Then, 5 μl of either binding buffer or fixed *S. aureus* cells (Pansorbin; Calbiochem) washed in binding buffer was added. After a final incubation of 30 min at 4°C, reaction mixes were centrifuged at 13,000 × *g* for 5 min at 4°C, and samples of the supernatants were electrophoresed. The apparent decrease in the ISGF1-DNA complex in samples that included immune serum is due to the decreased background around the original location of the abundant ISGF2-DNA complex formed in the EMSA with this extract. Free probe is not shown. The ISGF1, ISGF2, ISGF3, and ISGF2 immune complexes are indicated.

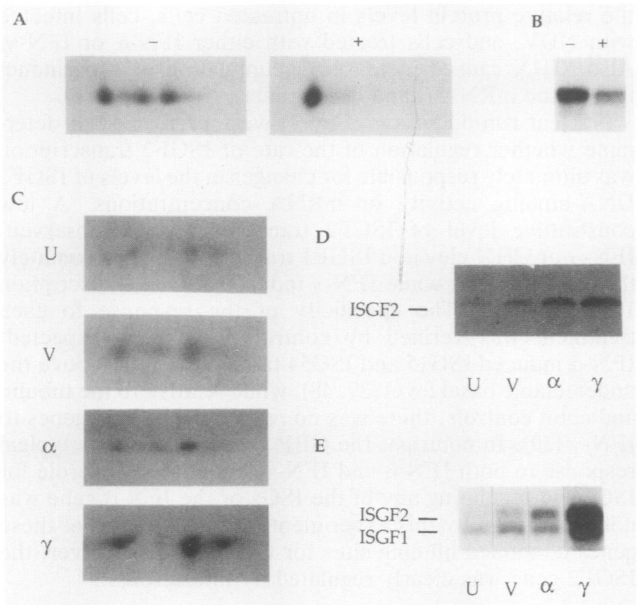

FIG. 6. Qualitative and quantitative characterization of basal and induced ISGF2. (A) Purified ISGF2 was incubated with BSA (0.1 mg/ml) for 1 h at 30°C in 50 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid, pH 6.0]–0.2 M NaCl–5 mM DTT–1.5 mM $MgCl_2$–10% (vol/vol) glycerol without (−) or with (+) 0.5 U of potato acid phosphatase (Sigma, type III) per ml, and then portions equivalent to 1.0 ng of ISGF2 were used for two-dimensional immunoblots. The area shown, corresponding to pI 5.3 to 4.7 (basic at left) and 50 to 60 kDa, contained the only immunoreactive material. (B) Portions equivalent to 0.2 ng of ISGF2 from the samples described in panel A were used for EMSA. Only the protein-DNA complex is shown. (C) Two-dimensional immunoblots were done with 100 μg of nuclear extract protein from cells that were (U) untreated, (V) 9 h after infection with NDV, (α) treated for 2 h with IFN-α (500 U/ml), or (γ) treated for 12 h with IFN-γ (1 ng/ml). Separate analyses of this and other extracts from IFN-γ-treated cells showed a distribution of ISGF2 isoforms equivalent to that seen in extracts from all other treated or untreated cells. The separations are shown with the basic end at the left. Only the area of pI 5.3 to 4.7 and 56 kDa is shown. (D) The extracts used for panel C were separated in one dimension by SDS-PAGE, and ISGF2 was detected by immunoblot analysis. Only the area around 56 kDa is shown. (E) The extracts used for panel C were analyzed by EMSA with an ISG15 ISRE oligonucleotide. Only the complexes formed by ISGF1 and ISGF2 are shown. The assay was performed with the ISG15 promoter probe and 5 μg of crude nuclear extract protein from untreated HeLa cells (lane 1), HeLa cells treated for 2 h with IFN-α (500 U/ml) (lane 2), or HeLa cells treated for 12 h with IFN-γ (1 ng/ml) (lane 3). Only the complexes formed by ISGF1 and ISGF2 are shown. The apparent induction of ISGF1 by IFN-γ is due to background around the extremely large amount of ISGF2.

ISGF2 has no demonstrable role in activation of IFN-β transcription. Recent reports have suggested a positive transcriptional role for ISGF2 (IRF-1) in regulating the IFN-β gene and possibly the ISGs. These conclusions rely on the significant effect of ISGF2 (IRF-1), expressed after transfection, on the expression of an artificial gene construct on a second plasmid. In these transfection experiments, the reporter gene contained a polymerized hexameric sequence found in the PRD-1 region of IFN-β and also in the ISRE. Only marginal effects were found on expression from the endogenous gene or a cotransfected IFN-β promoter construct (17, 21). In addition to the possible importance of the ISGF2 (IRF-1)-binding site in the IFN-β promoters, NFκB has also been shown to play a role in transcriptional activation of the IFN-β gene (18, 22, 32, 54).

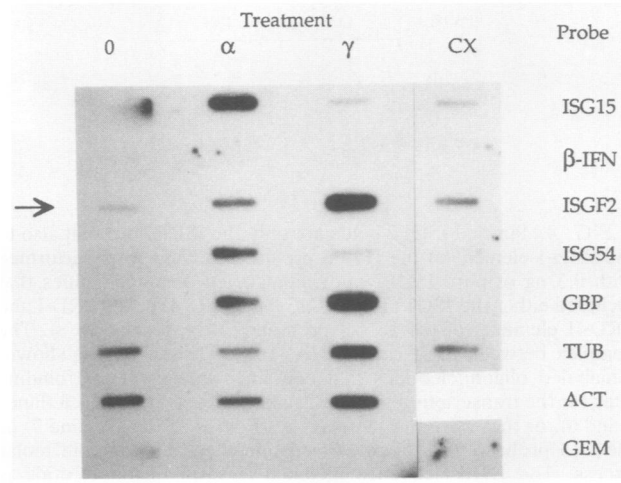

FIG. 7. ISGF2 is transcriptionally induced by IFN-α, IFN-γ, and CHX. Run-on transcription analysis was performed with nuclei isolated from untreated HeLa cells (lane 0) or HeLa cells that had been treated with IFN-α (500 U/ml) for 2 h, IFN-γ (5 ng/ml) for 2 h, or CHX (50 μg/ml) for 2 h, as indicated. Radiolabeled RNA was hybridized to excess DNA probes (see Materials and Methods) bound to nitrocellulose in the pattern shown. TUB, Tubulin; ACT, actin; GEM, pGEM1.

To assess the relative contribution the factors ISGF2 (IRF-1) and NFκB might make to transcription of the endogenous IFN-β gene, as opposed to artificial constructs assayed by transfection experiments (21), HeLa cells were treated with various combinations of dsRNA, to provide signal(s) for induction of IFN-β transcription; CHX, to induce NFκB and decrease the amount of ISGF2; and IFN-γ, to induce ISGF2. Nuclei were isolated both for run-on analysis of transcription rates of several genes and for preparation of protein extracts to test ISGF2 (IRF-1) and NFκB concentrations (Fig. 8). Two key results emerged from the experiment. Transcription of the IFN-β gene was strongly activated by treatment with dsRNA in the presence of CHX, circumstances under which NFκB was abundant and ISGF2 was undetectable (lane 3). In contrast, high levels of ISGF2 alone were not able to activate the IFN-β gene in the absence of NFκB (lane 7). The remaining results might indicate that when it was present, ISGF2 augmented activation by NFκB (compare lanes 5 and 3). However, other, unassayed factors may have been responsible for the increased transcription. In any case, IFN-β transcription was not induced unless a substantial amount of NFκB was present, regardless of the level of ISGF2 (lanes 2, 4, 6, and 7). In sum, these results show clearly that ISGF2 is neither necessary nor sufficient for transcriptional activation of the chromosomal IFN-β gene in HeLa cells.

DISCUSSION

The data in this article prove that the interferon-induced protein factor we have identified and termed ISGF2 (34, 35) is a single polypeptide that migrates on SDS-PAGE as a 56-kDa species (Fig. 1), even though the size predicted from the cDNA sequence is considerably smaller (see below). Renaturation of this single polypeptide produced the ISGF2 complex in an EMSA. Furthermore, the purified protein produced distinct footprints on the ISRE (Fig. 3), and

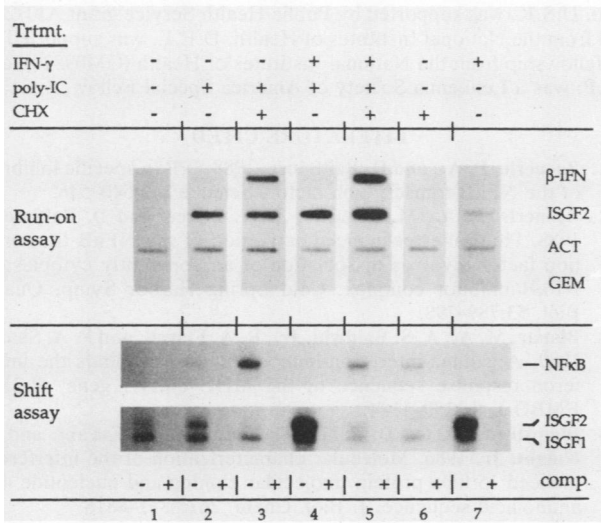

FIG. 8. ISGF2 is neither necessary nor sufficient for IFN-β gene transcription. HeLa cells were either untreated or treated with dsRNA (poly-IC, 50 μg/ml), CHX (50 μg/ml), or IFN-γ (5 ng/ml), singly or in combinations as shown. Treatments with IFN-γ were for 4 h. The other two reagents were added singly or together for 2 h or for the last 2 h of treatment with IFN-γ. Nuclei were isolated from the cells and used for run-on transcription analysis or to prepare protein extracts. Radiolabeled RNA from the transcription assays was hybridized to excess DNA probes (see Materials and Methods) bound to nitrocellulose in the pattern shown. ACT, Actin; GEM, pGEM1; comp., minus or plus specific competitor. An EMSA with the NFκB or O15 oligonucleotide probes described in Materials and Methods was performed with the nuclear extracts. A 200-fold molar excess of the respective unlabeled oligonucleotide was included as indicated (+). Only a single specific protein-DNA complex formed with the NFκB probe. It is identified based on published reports (32, 52). The ISGF1 and ISGF2 complexes with the O15 probe are indicated.

antibodies raised against this polypeptide affected specifically the ISGF2-DNA complex in an EMSA (Fig. 5).

Knowledge of the amino acid sequence from the amino terminus of purified ISGF2 plus the ISGF2-specific antibody allowed cloning of ISGF2 cDNAs. The sequence of the cDNAs revealed that these clones were the same as human IRF-1 (37). However, five bases differed from the previously published sequence (data not shown). Three of these discrepancies resulted in codon changes. Arginine instead of tryptophan would be encoded due to a C at position 210 instead of T; the arginine was found in the amino acid sequence of purified ISGF2 (unpublished data). The nucleotides at positions 299 and 300 were C C instead of G G, and the encoded amino acids would be phenylalanine glutamine instead of leucine glutamate. These three changes eliminated the only reported amino acid differences between mouse and human IRF-1 within the first 100 codons (40). The other two base changes, G G at positions 1676 and 1677 instead of C C, create an *Ava*II restriction site that has been confirmed. (Nucleotide positions are relative to the numbering of reference 40.)

The translation product of mRNA transcribed from an ISGF2 cDNA had the same apparent molecular weight as ISGF2 when analyzed by SDS-PAGE and was detected on an immunoblot by the anti-ISGF2 antibody, and the complex it formed with an ISG15 ISRE probe in an EMSA comigrated with that formed by ISGF2 (unpublished data). The predicted size of the human and mouse IRF-1 proteins was approximately 37 kDa, and the mouse protein was reported to migrate at that size on SDS-PAGE (40).

The bacterially expressed proteins encoded by mouse IRF-1, and the related cDNA IRF-2, bind to the PRD-1 element of the IFN-β promoter as well as the ISRE of the *H-2D*^{d} promoter (21, 40). The original mouse IRF-1 clone was obtained because it bound to a synthetic ISRE homolog created by ligation of a hexamer oligonucleotide (40). However, the correspondence of these clones to particular mouse proteins is not certain, and the DNA-binding characteristics of the individual mouse proteins are not known. In contrast, binding of authentic human ISGF2 to both the PRD-1 element of the human IFN-β promoter and the ISRE core in human ISG promoters (Fig. 4) actually proves that a single protein, human ISGF2, does bind to key regulatory sites in these genes.

ISGF2 is a phosphoprotein, and the affinity of site-specific DNA binding is dependent on the extent of phosphorylation (Fig. 6A and B). All the isoforms present in nuclear extracts were recovered by specific oligonucleotide affinity chromotography, and thus all the isoforms bound the ISRE. Elution that started at 0.25 M KCl but was only completed with 1.0 M KCl (data not shown) was consistent with different isoforms having different affinities for the ISRE. However, any importance for phosphorylation in ISGF2 function is unclear. The steady-state distribution of ISGF2 isoforms, which resulted primarily from differential phosphorylation, did not change significantly under different conditions where more or less ISGF2 existed in cells. Instead, changes in the abundance of ISGF2 seemed to determine the amount of its DNA-binding activity (Fig. 6C, D, and E). These data strongly suggest that ISGF2 protein induction per se would be the major contributor to its potential action. Furthermore, these results are inconsistent with one of several models proposed to account for the minimal induction of IRF-1 DNA-binding activity by virus infection, in which ISGF2 (IRF-1) regulation of IFN-β gene expression depends on posttranslational phosphorylation (16).

With the availability of the cDNA, we could show that the gene encoding ISGF2 is responsive to regulation by both IFN-α and IFN-γ at the transcriptional level; IFN-γ has a much stronger stimulatory effect than does IFN-α. Taniguchi and co-workers (17, 21) have concluded that IRF-1 is an important transcriptional activator of IFN-α and IFN-β gene expression and that the protein encoded by a related clone, IRF-2, is a repressor, based on cotransfection of expression plasmid constructs with a target plasmid containing a synthetic sequence that can mimic some regulatory properties of the IFN-β promoter (15). However, the IRF-1 and -2 expression constructs have little or no effect on a cotransfected target containing an intact IFN-β promoter or on endogenous IFN-β genes. Although various explanations were advanced to account for the discrepancy, the most obvious caveat is that results with artificial constructs might be artifacts, and thus IRF-1 or -2 might not be very significant in regulation of a chromosomal IFN-β gene.

In contrast to the clear correlation between the presence of ISGF3 and high transcription rates of ISGs (27, 34, 45), the presence of ISGF2 did not correlate with transcription of either ISGs or the IFN-β gene in HeLa cells. ISGF2 protein levels and DNA-binding activity are increased 3- to 5-fold by IFN-α and 10- to 20-fold by IFN-γ, yet there was no transcription of the chromosomal IFN-β genes under those circumstances (Fig. 6, 7, and 8). Moreover, dsRNA added to cells in the presence of CHX increased IFN-β transcription more than dsRNA alone, and under those circumstances the

concentration of ISGF2 (IRF-1) protein was actually below the trace uninduced level. In fact, IFN-β gene transcription is inducible in most cell types even if protein synthesis is inhibited (19).

The high level of NFκB induced by dsRNA plus CHX (Fig. 8) is probably due in part to inhibited synthesis of IκB (the cytoplasmic inhibitor of NFκB [1, 2]). The concomitant strong induction of IFN-β transcription is consistent with the established role of NFκB in transcriptional activation of the IFN-β gene (18, 22, 32, 54).

From our work on the IFN-α-stimulated transcription cycle of ISGs, we cannot ascribe a role to ISGF2 at present. After IFN-α treatment, ISG transcription promptly rises without protein synthesis (and therefore without induced ISGF2) and falls in a protein synthesis-dependent manner. The fall in transcription therefore correlated with induced synthesis of ISGF2 (34). However, ISGF2 induced by IFN-γ treatment of FS-2 or HeLa cells does not seem to reduce induction of ISGs by IFN-α, and in HeLa cells superinduction actually occurs (D. E. Levy, D. J. Lew, T. Decker, D. S. Kessler, and J. E. Darnell, Jr., EMBO J., in press). So the presence of large amounts of ISGF2 does not guarantee a negative effect on ISG transcription.

We thus arrive at a situation where there is a clearly identified, site-specific DNA-binding protein whose concentration changes during induction of cells with IFN-α or IFN-γ or by treatment of cells with dsRNA. Furthermore, it binds not only to the promoter of other genes that are also induced by interferons, but also to a key regulatory element of the IFN-β gene. However, there is no clear evidence of the physiological role of ISGF2 (IRF-1). The presence of the protein has not been proven to cause significant positive or negative transcriptional effects on any chromosomal gene that has a known ISGF2-binding site.

Does this mean it has no effect? Certainly not, but it suggests that the protein's behavior in transfection experiments with artificial target constructs is not a reliable indicator of how it behaves in vivo. Unfortunately, in vitro transcription with purified proteins may be subject to the same reservations. While results from manipulation of cell culture conditions can be interpreted to reveal all-or-none effects, less obvious outcomes of complex interactions can be difficult to discern.

ISGF2 may be part of a regulatory network that is activated by IFN treatment. Its role could be to modulate transcription of genes whose primary activation depends on other factors, or it could be the primary activator of genes that are secondary participants in the cellular response to IFN. For example, there is some evidence that a protein which may be ISGF2 is involved in regulation of class I histocompatibility antigen genes (3). Perhaps gene knock-out experiments are the only way to pinpoint the role of a binding protein which may be a subtle second-order regulatory molecule in the cascade of events that is begun when cells are exposed to interferon.

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